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Immunoproteomic analysis of the antibody response obtained in Nile tilapia following vaccination with a *Streptococcus iniae* vaccine

Benjamin R. LaFrentz *, Craig A. Shoemaker, Phillip H. Klesius

USDA-ARS, Aquatic Animal Health Research Unit, 990 Wire Road, Auburn, AL 36832-4352, USA

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ABSTRACT

Streptococcus iniae is one of the most economically important Gram-positive pathogens in cultured fish species worldwide. The USDA-ARS Aquatic Animal Health Research Unit developed a modified (contains concentrated culture supernatant) *S. iniae* bacterin that has been demonstrated to be efficacious, and protection is mediated by specific anti-*S. iniae* antibodies. Although effective, the specific vaccine components important for efficacy are not known. In the present study, an immunoproteomic approach was utilized to identify whole-cell lysate proteins of *S. iniae* that stimulated specific antibody production in Nile tilapia (*Oreochromis niloticus*) following vaccination. Groups of tilapia were vaccinated by intraperitoneal injection with the modified *S. iniae* bacterin or were mock-vaccinated, and at 30 d post-vaccination sera samples were obtained from individual fish. Vaccination of tilapia with the *S. iniae* vaccine stimulated significantly elevated specific antibody responses against proteins of the bacterium and passive immunization of tilapia with this serum demonstrated the antibodies were highly protective. Whole-cell lysate proteins of *S. iniae* were separated by 2D-PAGE and were probed with a pooled serum sample from vaccinated tilapia. A total of eleven unique immunogenic proteins were positively identified by mass spectrometry. Based on research conducted on homologous proteins in other *Streptococcus* spp., antibodies specific for three of the identified proteins, enolase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase, are likely involved in protection from streptococcosis caused by *S. iniae*.

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1. Introduction

Streptococcus iniae is an etiologic agent of streptococcal disease in fish and is emerging as one of the most important Gram-positive bacterial fish pathogens impacting worldwide aquaculture production. Twenty-seven fish species have been reported to be susceptible (Agnew and Barnes, 2007), and due to a global distribution, this number is likely to increase as demonstrated by a recent report of the bacterium in a new cultured fish species, red porgy *Pagrus pagrus* (El Aamri et al., 2010). The estimated annual impact of *S. iniae* infections on the aquaculture industry in the United States is $10 million and estimated worldwide to be $100 million (Shoemaker et al., 2001).

In response to this problem, the USDA-ARS Aquatic Animal Health Research Unit (AAHRU) developed a modified *S. iniae* bacterin comprised of concentrated culture supernatant and formalin killed whole-cells (Klesius et al., 1999, 2000). The vaccine is consistently efficacious in Nile tilapia (*Oreochromis niloticus*) by intraperitoneal injection with relative percent survivals ranging from 80 to 100% following homologous (Klesius et al., 1999) and heterologous (Shoemaker et al., 2010) isolate challenges. Tilapia exhibit significantly elevated antibody titers following vaccination (Klesius et al., 2000; Shoemaker et al., 2010), and a protective role of specific

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail address: benjamin.lafrentz@ars.usda.gov (B.R. LaFrentz).

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antibodies was demonstrated by passive immunization experiments (Eldar et al., 1997; Shelby et al., 2002a). The concentrated supernatant induces a chemotactic response in macrophages (Klesius et al., 2007) and this stimulation of the proinflammatory response likely contributes to an effective immune response. Although the vaccine is effective, the specific components important for efficacy are not known. Therefore, the objective of this study was to use an immunoproteomic approach to identify proteins of *S. iniae* that are immunogenic in tilapia vaccinated with the modified *S. iniae* bacterin.

2. Materials and methods

2.1. Fish and rearing conditions

Two groups of Nile tilapia with mean weight of 49.9 ± 2.3 g and 22.3 ± 3.4 g were obtained from healthy stocks maintained at the USDA-ARS AAHRU. Necropsy was performed on ten fish from each group, and brain and head kidney samples were plated onto sheep blood agar (SBA; Remel, Lenexa, KS, USA) and incubated at 28 °C for 72 h to verify fish were free of *S. iniae*. None of the fish sampled were culture positive for the bacterium. Tilapia were acclimated for 10 d prior to experiments in 57 l aquaria supplied with 28 °C de-chlorinated municipal water and fed 3% body weight per day with Aquamax Grower 400 (PMI Nutrition International, LLC, Brentwood, MO, USA). All procedures utilizing fish were approved by the USDA-ARS AAHRU Institutional Animal Care and Use Committee.

2.2. Production of antiserum

The modified *S. iniae* bacterin, comprised of *S. iniae* ARS-98-60, was prepared as described by Klesius et al. (1999, 2000). Two tanks of fifteen Nile tilapia (mean weight, 49.9 ± 2.3 g) were used. Fish were anaesthetized in 90 mg l^{-1} tricaine methanesulfonate (MS-222, Argent Chemicals, Redmond, WA, USA) and vaccinated fish were injected intraperitoneally (ip) using a 26-guage needle with 100 μl of the vaccine. Fish in the mock-vaccinated control group were injected ip with 100 μl of sterile tryptic soy broth (TSB).

At 30 d post-vaccination, serum samples were collected from individual tilapia in the treatment and control groups. Fish were euthanized with an overdose of MS-222 and bled by caudal puncture using a 25-guage needle. Blood was transferred to microcentrifuge tubes, allowed to clot for 2 h at room temperature and centrifuged at $8000 \times g$ for 10 min. Serum was collected and stored at −20 °C.

2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were performed on all serum samples to quantify anti-*S. iniae* antibodies using a method modified from Shelby et al. (2002b). Nunc Maxisorp® flat-bottom 96 well plates (Nalge Nunc International, Rochester, NY, USA) were coated with 100 μl *S. iniae* antigen, diluted in carbonate buffer to 25 μg ml^{-1}, and allowed to bind overnight at 4 °C. Plates were washed three times with phosphate buffered saline containing 0.05% tween-20 (PBST), then 100 μl of diluted (1:1000 in PBST) serum samples in triplicate were added to the wells and incubated for 2 h at room temperature. Negative and positive serum samples were included as assay controls. Plates were washed, then 100 μl of diluted (1:1000 in PBST) monoclonal anti-tilapia immunoglobulin (MAb 1H1; Shelby et al., 2002b) was added to each well and incubated for 1 h at room temperature. Following washing the plates, rabbit anti-mouse IgG horseradish peroxidase conjugate (Sigma–Aldrich, St. Louis, MO, USA) was diluted 1:5000 in PBST and 100 μl was added to each well and incubated for 1 h at room temperature. Plates were washed, then 50 μl of 1-Step™ Ultra TMB-ELISA solution (Pierce, Rockford, IL, USA) was added to each well, incubated for 15 min at room temperature, and then the reaction was stopped by adding 50 μl of 3 M sulfuric acid to each well. The optical density (OD) of each well at 450 nm was determined using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Passive immunization

One hundred Nile tilapia (mean weight, 22.3 ± 3.4 g) were randomly allocated to ten 57 l tanks. Tilapia in triplicate tanks were passively immunized by ip injection (26 gauge needle) with 100 μl of control serum, antiserum, or sterile phosphate buffered saline (PBS). The control serum and antiserum were prepared by pooling equal volumes of sera from mock-vaccinated tilapia or sera from tilapia vaccinated with the modified *S. iniae* bacterin, respectively (see Section 2.2). Prior to use, the serum samples were heat inactivated for 1 h at 56 °C. Tilapia in the remaining tank were mock-immunized by ip injection with 100 μl sterile PBS. At 24 h post-passive immunization, passively immunized fish (control serum, antiserum, and PBS) were challenged with *S. iniae* by ip injection of 100 μl containing 6.6×10^5 colony-forming units per fish. Tilapia in the mock-immunized group were mock-challenged by ip injection with 100 μl sterile TSB. Mortalities were recorded twice daily for 16 d and brain tissue from all mortalities was plated onto SBA to reisolate *S. iniae*. The mean cumulative percent mortality (CPM) was calculated for each group and the relative percent survival (RPS) was determined (Amend, 1981).

2.5. Bacterial culture and preparation of whole-cell lysate

A virulent strain of *S. iniae*, ARS-98-60 (Klesius et al., 2000), was used for extraction of whole-cell lysate proteins. The isolate was recovered from a frozen glycerol stock, inoculated into TSB, and cultured for 24 h at 28 °C with shaking (125 rpm). Cells were harvested by centrifugation at $5000 \times g$ for 15 min at 4 °C. The supernatant was removed and aliquots of cells (~100 mg, wet weight) were frozen at −80 °C. Whole-cell lysates were prepared as described by Shoemaker et al. (2010) and stored at −80 °C until needed for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2.6. SDS-PAGE and Western blot analysis

Whole-cell lysate proteins of *S. iniae* were subjected to SDS-PAGE as described by LaFrentz et al. (2004). Proteins

(25 μg) were separated in precast 12% polyacrylamide gels using a Mini-Protean Tetra Cell (Bio-Rad) and Precision Plus protein standards (Bio-Rad) were included. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and Western blotting was performed as described by Shoemaker et al. (2010). Transferred proteins were probed with antibodies from eight individual serum samples of vaccinated tilapia and a pool of serum from mock-vaccinated tilapia as a negative control.

2.7. 2D-PAGE

Whole-cell lysate proteins were diluted into rehydration buffer containing 8 M urea, 50 mM dithiothreitol (DTT), 4% CHAPS, and 0.25% Bio-Lyte 3/10 ampholyte (Bio-Rad). Protein samples were vortexed for 1 min, incubated at room temperature for 2.5 h, then centrifuged at 10 000 × *g* for 10 min at room temperature. Immobilized pH gradient (IPG) strips (11 cm, pH 4–7; Bio-Rad) were passively rehydrated with 200 μl rehydration buffer containing 40 μg protein for 1 h, covered with mineral oil, and then rehydrated overnight at room temperature. First dimension isoelectric focusing was performed using a PROTEAN IEF Cell (Bio-Rad). IPG strips were focused at 250 V for 15 min, 8000 V for 2.5 h, 8000 V for 35,000 volt hours (Vh), and then held at 500 V until frozen at −80 °C. Focused strips were thawed and equilibrated for 20 min in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8, 20% glycerol) containing 130 mM DTT and then equilibrated for an additional 20 min in equilibration buffer containing 135 mM iodoacetamide. Second dimension separation of equilibrated IPG strips was performed in 4–20% linear gradient polyacrylamide gels (Bio-Rad) using a Criterion™ cell and Precision Plus (Bio-Rad) or Li-Cor Biosciences protein standards were included. Gels were electrophoresed using the standard Laemmli buffer system (Laemmli, 1970) for 20 min at 90 V and then at 200 V until the dye front migrated to the bottom of the gels. Gels were either stained with SYPRO Ruby (Bio-Rad) and imaged or used in Western blot analyses.

2.8. 2D-PAGE Western blot analyses

Proteins resolved by 2D-PAGE were subsequently transferred to nitrocellulose membranes for Western blot analysis by electrophoresis at 100 V for 1 h in a Criterion™ blotter (Bio-Rad) according to manufacturer's directions. Membranes were blocked for 1 h in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA), then a pooled serum sample from vaccinated tilapia (pool of equal volumes of sera from fifteen individual fish) was diluted (1:40 in Odyssey blocking buffer with 0.1% tween-20), applied to the membranes, and incubated overnight at 4 °C. Membranes were washed four times with PBS containing 0.1% tween-20, then MAb 1H1 was diluted 1:500 into Odyssey blocking buffer with 0.1% tween-20, applied to the blots, and incubated for 1 h at room temperature. Membranes were washed four times in PBST, then IRDye® 680 conjugated goat anti-mouse IgG (Li-Cor Biosciences) was diluted 1:5000 into Odyssey blocking buffer with 0.1% tween-20, applied to the blots and incubated for 1 h at room temperature. Membranes were washed 4 times in PBS containing 0.1% tween-20, two times in PBS, and then imaged with an Odyssey® infrared imaging system (Li-Cor Biosciences). Western blots were performed in triplicate and consistent results were obtained.

Following Western blot analyses, immunogenic proteins were matched to the corresponding proteins in SYRPO Ruby stained gels and then manually excised. Excised proteins were placed in 5% acetic acid and submitted to the University of Alabama at Birmingham Targeted Metabolomics and Proteomics Laboratory (Birmingham, AL, USA) for LC–MS/MS analysis.

2.9. LC–MS/MS analysis

Proteins within each excised gel piece were subjected to in-gel tryptic digestion (Shevchenko et al., 1996). Aliquots (5–10 μl) of each digest were automatically injected by a CTC PAL autosampler (Leap Technologies, Carrboro, NC, USA) onto a C_{18} reverse-phase cartridge (5 mm × 100 μm; Phenomenex, Torrance, CA, USA) at 20 μl min^{-1} and then washed for 5 min with 0.1% formic acid. The bound peptides were then transferred onto a C_{18} reverse-phase pulled tip analytical column (Phenomenex). Peptides were eluted from this column over 30 min with a linear 5–50% acetonitrile gradient in 0.1% formic acid at 500 nl min^{-1}. The eluted peptides were passed directly from the tip into a modified MicroIonSpray interface of a 4000 Qtrap mass spectrometer (Applied Biosystems-MDS-Sciex, Concorde, Ontario, Canada). Eluted peptides were subjected to a survey scan to determine the top three ions followed by a second enhanced resolution scan to determine the charge state of the selected ions. Enhanced product ion scans were then performed to obtain the tandem mass spectrum of the selected parent ions. Spectra were centroided and de-isotoped by Analyst Software, version 1.42 (Applied Biosystems, Foster City, CA). The mass spectrometry data were searched against the Gram-positive NCBInr protein database using the Mascot searching algorithm (Matrix Science). For all search inquiries, the parent mass tolerance was set to 100 ppm, fragment tolerance was 0.8 Da, 1 tryptic missed cleavage was allowed, and the carbamidomethylation of cysteines and oxidation of methionines were considered. Identifications were considered positive if two or more peptides from the same protein were matched and each matched peptide had an individual Mascot score greater than 40. Estimation of the theoretical molecular mass (M_r) and isoelectric points of the identified proteins were determined using the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

2.10. Statistical analyses

The ELISA OD values from vaccinated and mock-vaccinated tilapia were analyzed by a Student's *t*-test. The mean CPM of fish passively immunized with control serum, antiserum, or PBS was analyzed by a one-way analysis of variance with Tukey's test for pair wise comparisons. Differences were considered significant when $P < 0.05$. Data were analyzed and graphically

represented using GraphPad Prism (version 5.03, GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Production of antiserum

Vaccination of tilapia with the modified *S. iniae* bacterin resulted in the induction of specific antibody responses against the bacterium. The mean ELISA OD for vaccinated tilapia was significantly ($P < 0.05$) higher than the mock-vaccinated controls (Fig. 1). The ELISA results were confirmed by Western blot analysis of SDS-PAGE separated proteins, which demonstrated that vaccinated tilapia exhibited antibodies specific for *S. iniae* proteins (Fig. 2). Vaccinated fish exhibited antibodies specific for numerous proteins with approximate molecular masses of 25–125 kDa and there was variability in number and sizes of immunogenic proteins detected among individual sera samples. Negative control sera from mock-vaccinated fish exhibited weak reactivity to a few proteins and a protein band at approximately 49 kDa (Fig. 2).

3.2. Passive immunization

Tilapia passively immunized with the antiserum exhibited significantly ($P < 0.05$) lower CPM following *S. iniae* challenge compared with fish passively immunized with the control serum or PBS. CPM averaged 0, 70, and 45% for fish passively immunized with the antiserum, control serum, or PBS, respectively (Fig. 3). A RPS value of 100% was determined for fish passively immunized with the antiserum. There were no significant differences in the CPM between fish passively immunized with the control serum or PBS. No mortality occurred in the mock-immunized/mock-challenged group and *S. iniae* was re-isolated from all mortalities.

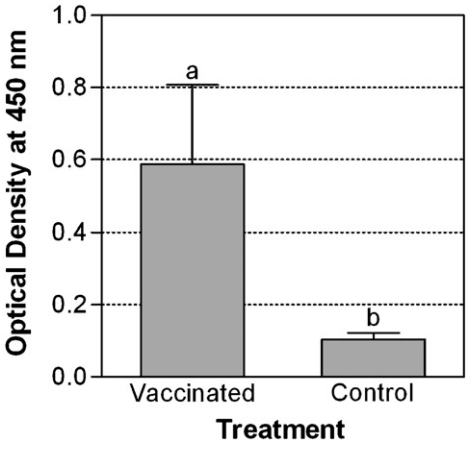

Fig. 1. Specific anti-*S. iniae* antibody levels in Nile tilapia 30 d post-vaccination with a modified *S. iniae* bacterin or mock-vaccinated (control) as measured by mean ELISA optical density values. Data are presented as the mean optical density ± standard deviation obtained from ELISA analysis of individual serum samples ($n = 15$ per treatment) at a 1:1000 dilution. Bars with different letters indicate a significant difference at $P < 0.05$.

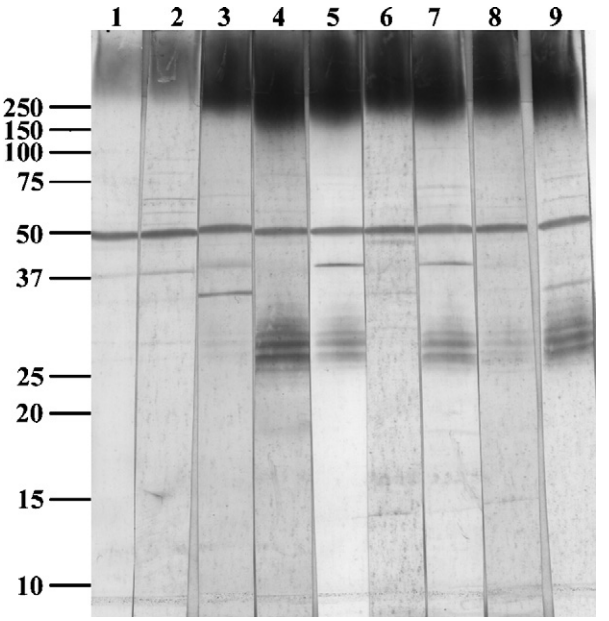

Fig. 2. Western blot analysis of whole-cell lysate proteins of *S. iniae*. Proteins were probed with pooled serum antibodies from mock-vaccinated tilapia (Lane 1) and serum antibodies from individual Nile tilapia vaccinated with a modified *S. iniae* bacterin (Lanes 2–9). Molecular mass markers (kDa) are indicated to the left of the blot.

3.3. Identification of immunogenic proteins

Numerous immunogenic protein spots were detected in the 2D-PAGE Western blots with approximate molecular masses ranging from 25 to 100 kDa (Fig. 4), and a total of thirty-three immunogenic protein spots could be confidently matched to the corresponding protein spot in SYPRO ruby stained gels. Of these, seventeen protein spots that exhibited the strongest fluorescent signal from the Western blot were excised for identification (boxed in Figs. 4 and 5). Single proteins were positively identified for sixteen of these, but only eleven of these were unique

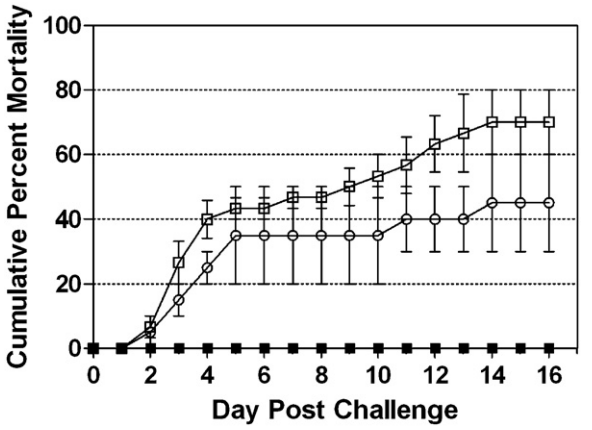

Fig. 3. Mean cumulative percent mortality of Nile tilapia following challenge with *S. iniae*. Fish were passively immunized with control serum (□), antiserum (■), or PBS (○). Error bars indicate standard error of the mean.

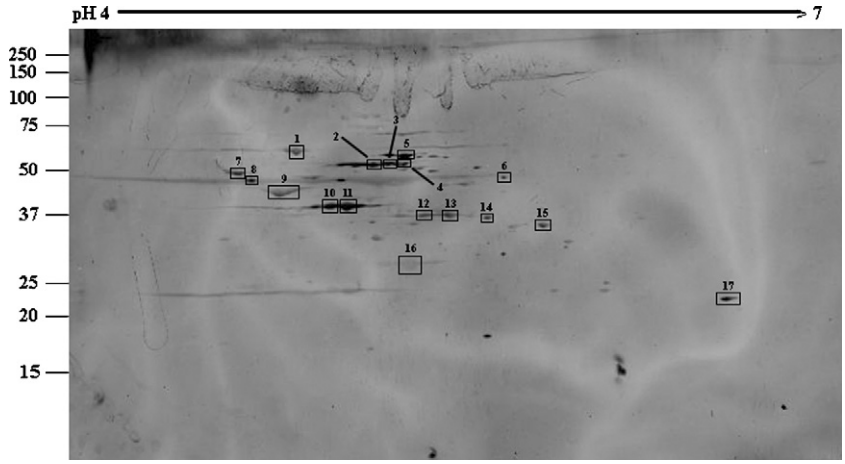

Fig. 4. 2D-PAGE Western blot of whole-cell lysate proteins of *S. iniae*. Proteins were probed with serum antibodies from Nile tilapia vaccinated with a modified *S. iniae* bacterin. Protein spot numbers indicate immunogenic proteins that could be confidently matched to stained gels (Fig. 5, Table 1). Molecular mass markers (kDa) are indicated to the left of the blot.

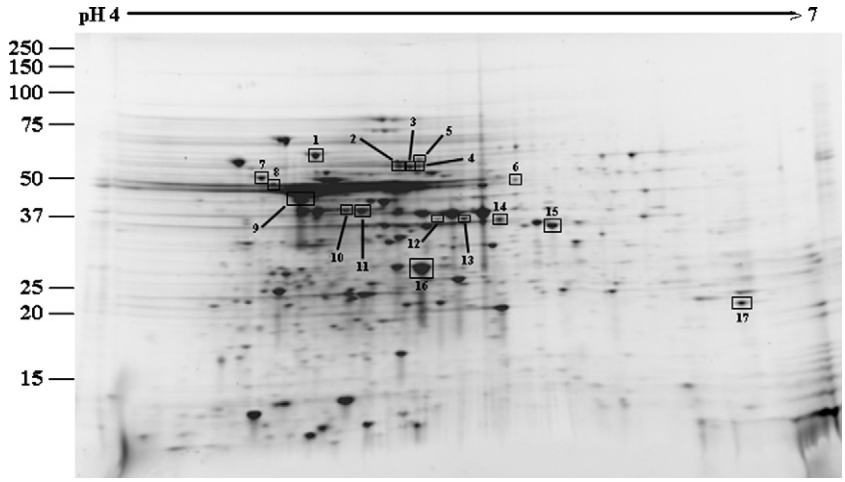

Fig. 5. 2D-PAGE analysis of whole-cell lysate proteins from *S. iniae*. Proteins were visualized by Sypro Ruby staining. Protein spot numbers correspond to immunogenic proteins in Fig. 4 and were identified by LC–MS/MS (Table 1). Molecular mass markers are indicated to the left of the gel.

because several of the protein spots were identified as the same protein (Table 1). Two proteins were positively identified in Protein spot no. 15 (Table 1).

4. Discussion

Immunity against streptococcosis caused by *S. iniae* has been demonstrated to be mediated by specific antibodies produced by the host (Eldar et al., 1997; Shelby et al., 2002a). Although this is known, there is little information regarding the components of the bacterium these antibodies are directed against. A few studies have demonstrated *S. iniae* proteins are immunogenic (Eldar et al., 1997; Shin et al., 2007; Shoemaker et al., 2010), but the identity of these was not determined. In the present study, control serum and antiserum were generated in Nile tilapia following mock-vaccination or vaccination with a modified *S. iniae* bacterin, respectively. Similar to previous studies with this vaccine (Klesius et al., 2000; Shoemaker et al., 2010), vaccinated fish generated elevated specific antibody responses. A passive immunization study was performed to verify that the antiserum contained protective antibodies and the results confirmed this (RPS of 100%). These initial experiments demonstrated that the antiserum was appropriate for an immunoproteomic analysis. Subsequently, immunogenic proteins were identified by probing whole-cell lysate proteins of *S. iniae* with the antiserum. Based on research conducted on homologous proteins in other *Streptococcus* spp., some of the identified proteins are likely important for vaccine efficacy and this information increases our understanding of the acquired immune response of tilapia against *S. iniae*.

The enolase (EN), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and fructose-bisphosphate aldolase (FBA) proteins of *S. iniae* were identified as immunogenic in the present study. This is a common finding among *Streptococcus* spp. (Ling et al., 2004; Cole et al., 2005; Zhang and Lu, 2007; Geng et al., 2008; Mao et al., 2008; Wu et al., 2008; Zhang et al., 2008), and there are data to support a role of these as protective antigens. Experimental vaccines,

Table 1
Immunogenic proteins of *S. iniae* identified by LC–MS/MS. Protein spot numbers refer to proteins in Figs. 4 and 5.

Protein spot no.	Identified protein	Species	NCBI accession no.	Theoretical M_r (kDa)/pI	No. peptides matched	Sequence coverage (%)	Mascot score
1	Chaperonin GroEL	*S. agalactiae* (2603V/R)	gi|22538209	57.296/4.69	19	34	1077
2	Pyruvate kinase	*S. pyogenes* M1 GAS	gi|15675235	54.558/4.96	10	24	579
3	Pyruvate kinase Pyk	*S. equi* subsp. *zooepidemicus* MGCS10565	gi|195978009	54.638/5.09	9	20	501
4	Pyruvate kinase	*S. pyogenes* M1 GAS	gi|15675235	54.558/4.96	8	14	342
5	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	*S. uberis* 0140J	gi|222153239	50.247/5.07	2	4	108
6	Serine hydroxymethyltransferase	*S. pyogenes* M1 GAS	gi|15675122	45.182/5.68	2	6	154
7	Cell division protein FtsZ	*S. uberis* 0140J	gi|222153417	46.763/4.55	8	27	729
8	Cell division protein FtsZ	*S. uberis* 0140J	gi|222153417	46.763/4.55	6	13	394
9	Enolase	*S. uberis* 0140J	gi|222152817	47.335/4.64	21	61	1570
10	DNA-directed RNA polymerase subunit alpha	*S. mutans* UA159	gi|24380343	34.606/4.76	9	27	441
11	DNA directed RNA polymerase alpha subunit	*S. agalactiae* (2603V/R)	gi|22536269	34.551/4.84	13	38	768
12	Glyceraldehyde-3-phosphate dehydrogenase GapC	*S. agalactiae*	gi|21666599	35.815/5.22	6	19	265
13	Glyceraldehyde-3-phosphate dehydrogenase GapC	*S. agalactiae*	gi|21666599	35.815/5.22	2	13	161
14	PTS system mannose-specific EIIAB component ManX	*S. equi* subsp. *zooepidemicus* MGCS10565	gi|195977564	35.361/5.38	5	19	237
15	Ribose-phosphate pyrophosphokinase Prs	*S. equi* subsp. *zooepidemicus* MGCS10565	gi|195978168	35.487/5.91	3	9	154
	6-Phosphofructokinase	*S. uberis* 0140J	gi|222153150	35.617/5.78	2	10	81
16	Fructose-bisphosphate aldolase	*S. pyogenes* M1 GAS	gi|15675705	31.303/4.87	6	14	376
17	Ribosome recycling factor Frr	*S. equi* subsp. *zooepidemicus* MGCS10565	gi|195978553	20.542/5.87	5	21	272

comprised of recombinant EN, FBA and GAPDH proteins, have been shown to provide protective immunity against *Streptococcus* spp. with protection likely mediated by specific antibodies (Fontaine et al., 2002; Ling et al., 2004; Dinis et al., 2009; Feng et al., 2009). Although these are typically considered cytoplasmic proteins involved in glycolysis, they are often cell-wall associated (Hughes et al., 2002; Ling et al., 2004; Cole et al., 2005; Severin et al., 2007; Zhang and Lu, 2007; Geng et al., 2008; Wu et al., 2008; Zhang et al., 2008) and virulence factors with roles in adhesion, colonization, and tissue invasion (Pancholi and Fischetti, 1992; Blau et al., 2007; Kim et al., 2007). Thus, the effector mechanisms of specific antibodies against these proteins may include blocking the ability of the bacteria to adhere to and invade host tissues, opsonization of bacterial cells and stimulating phagocytosis by macrophages, and activation of the complement cascade. In addition to other *Streptococcus* spp., there is data to support a role of these proteins in protective immunity against *S. iniae*. Shin et al. (2007) suggested that the EN, FBA, and GAPDH proteins of *S. iniae* are immunogenic in olive flounder (*Paralichthys olivaceus*), although the proteins were not definitively identified. Kim et al. (2007) identified the enolase of *S. iniae* as a potential target for a subunit vaccine due its surface exposure and likely role in invading host tissues. Additionally, a protective role of the GAPDH protein of *S. iniae* was suggested following vaccination of fish with ghost bacteria expressing recombinant GAPDH (Ra et al., 2010). Based on these findings, specific antibodies against EN, FBA, and GAPDH are likely involved in the protection conferred to Nile tilapia by vaccination with the modified *S. iniae* bacterin.

Several proteins identified in this study have been previously determined to be immunogenic in other *Streptococcus* spp., including pyruvate kinase, PTS system mannose-specific EIIAB component ManX, ribose-phosphate pyrophosphokinase Prs, 6-phosphofructokinase, DNA-directed RNA polymerase, and ribosome recycling factor Frr (Cole et al., 2005; Geng et al., 2008; Mao et al., 2008; Wu et al., 2008; Wang et al., 2011). Information concerning the protective nature of these is lacking; however, each has been suggested to be cell-wall associated in other *Streptococcus* spp. (Cole et al., 2005; Severin et al., 2007; Geng et al., 2008; Wu et al., 2008; Wang et al., 2011). Should these proteins also be cell-wall associated in *S. iniae*, it is conceivable that antibodies specific for them may be involved in protective immunity. The remaining proteins identified in this study have not been previously demonstrated to be immunogenic in other *Streptococcus* spp., but may represent important proteins for acquired immunity against *S. iniae*.

To attain knowledge of the protective components of the modified *S. iniae* bacterin, this study initially focused on whole-cell lysate proteins and several likely candidates

were identified, namely EN, FBA, and GAPDH. However, the vaccine also contains concentrated culture supernatant in addition to killed cells. Thus, other vaccine components, such as extracellular products and carbohydrates, may be important. Studies have demonstrated that incorporating extracellular products into formalin-killed *S. iniae* vaccines enhances protection against experimental streptococcosis (Klesius et al., 1999; Shin et al., 2007). Additionally, a protective role of carbohydrate components of the bacterium has been suggested (Eyngor et al., 2008). Future research will examine these in order to gain a comprehensive understanding of the protective components of the modified *S. iniae* bacterin.

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